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Hemagglutinin Receptor Specificity and Structural Analyses of Respiratory Droplet-Transmissible H5N1 Viruses

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Two ferret-adapted H5N1 viruses capable of respiratory droplet transmission have been reported with mutations in the hemagglutinin receptor-binding site and stalk domains. Glycan microarray analysis reveals that both viruses exhibit a strong shift toward binding to "human-type" α2-6 sialosides but with notable differences in fine specificity. Crystal structure analysis further shows that the stalk mutation causes no obvious perturbation of the receptor-binding pocket, consistent with its impact on hemagglutinin stability without affecting receptor specificity.

H5N1 influenza A virus is of avian origin and is spread by migrating waterfowl. It is highly pathogenic for domestic poultry, causing economic problems as a result of periodic outbreaks. Although human infections are sporadic, they are accompanied by high mortality, raising major concerns about the potential of H5N1 as a pandemic virus (1, 2). Fortunately, H5N1 viruses have not yet naturally acquired the ability to stably transmit between humans (3, 4). One factor that limits transmission of avian viruses in humans is the receptor specificity of the hemagglutinin (HA) (5). Avian viruses, like H5N1, preferentially bind to α2-3 sialosides (avian-type receptors), whereas human viruses prefer α2-6 sialosides (human-type receptors that are found in the human respiratory tract). The three influenza A virus serotypes that have circulated in humans to date (H1N1, H2N2, and H3N2) are believed to have evolved from avian origin and to have acquired the ability to recognize α2-6 sialosides through minimal mutations in the receptor binding pocket of the HA (5, 6). Since binding to α2-6 sialosides is believed to be vital for efficient transmission between humans, there has been a major effort to define how H5N1 viruses could acquire this ability (6, 7).

Recently, two groups evaluating adaptation of H5N1 viruses in ferrets reported respiratory droplet-transmissible variants with mutations in the receptor-binding site (RBS) and stalk domain (5, 8). Ferrets have become the accepted animal model for transmission of human influenza viruses, in part because their upper airway epithelium is enriched in human-type receptors, as evidenced by preferential binding of human influenza viruses and plant lectins specific for α2-6 sialosides (9, 10). Viruses with the H5 HAs from A/Vietnam/1203/04 (VN1203) and A/Indonesia/5/05 (INDO5) backgrounds were initially engineered to contain mutations in the RBS known to increase avidity with respect to α2-6 sialosides and then acquired additional mutations that facilitated respiratory droplet transmission among ferrets during serial passage in ferret lungs. During ferret passage, the HA of both viruses lost an N-linked glycan at N158 on the edge of the RBS and acquired an additional mutation in the stalk. In this study, we sought to analyze the impact of these additional mutations on the receptor specificity and structure of the H5 HA.

Glycan array analysis has become a standard assay to assess influenza A virus receptor specificity (6, 11, 12), since it probes numerous biologically important glycans within a single assay. Glycan array analysis is a qualitative assay, and it has been shown that graded dilution of the binding protein reduces the signal without significantly altering the relative levels of binding to different glycans (13). We examined the VN1203 H5 mutants in the Cal/04 (A/California/04/2009 [H1N1]) virus background as inactivated whole virus (VN1203/7xCal/04/09 virus) and also evaluated recombinant, soluble, and trimeric HA proteins produced in mammalian 293T HEK GnTI(−) cells (14) for mutants of the VN1203 and INDO5 strains. We applied the viruses and HAs to the glycan array containing 55 sialosides with terminal α2-3 and α2-6 sialic acids (15, 16). Viruses were subjected to the array at 512 hemagglutinating units (HAU), incubated for 1 h at 4°C, and detected with polyclonal serum (17). For recombinant proteins, HA-antibody complexes were first prepared by mixing 50 μg/ml, or 150 μg/ml, of the HA mutants with mouse anti-streptomycin-horseradish peroxidase (anti-Strep-HRP) (IBA) and anti-mouse-IgG-Alexa Fluor 488 (Invitrogen) in a molar ratio of 4:2:1 (18), with the exception of the VN1203 T318I HA, which was obtained from expression in insect cells, and mouse anti-His-Alexa 488 was used as the primary antibody in the HA-antibody complex, as previously described (15, 16).

A human control A/Kawasaki/173/07 (H1N1) virus bound

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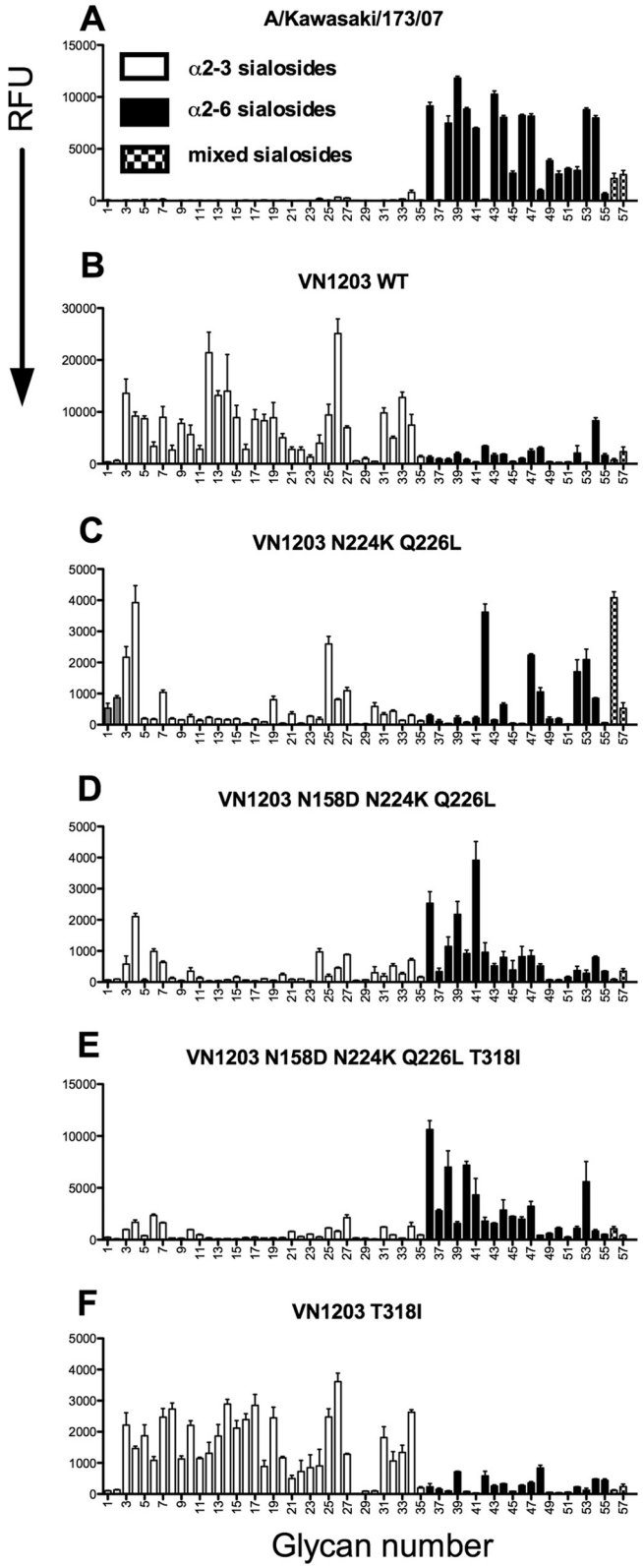

FIG 1 Glycan microarray analyses of respiratory droplet-transmissible H5N1 virus. The receptor specificity of whole inactivated VN1203/7xCal/04/09 virus with WT and mutant H5 HAs was assessed using glycan microarrays. Glycan microarrays comprised nonsialylated controls (gray bars; glycan no. 1 and 2 on the *x* axis), α2-3-linked sialosides (white bars; 3 to 35 on the *x* axis), α2-6-linked sialosides (black bars; 36 to 55 on the *x* axis), and glycans with both α2-3- and α2-6-linked sialic acids (checkered bars; 56 and 57 on the *x* axis). The *y* axis represents relative fluorescence units (RFU). The data shown represent control human virus (A/Kawasaki/173/07 [H1N1]) (A), WT VN1203 virus (B), VN1203 N224K Q226L HA mutant virus (C), VN1203 N158D N224K Q226L HA mutant virus (D), VN1203 N158D N224K Q226L T318I HA mutant virus (E), and VN1203 T318I HA mutant virus (F). A complete list of glycan structures can be found in Table S1 in the supplemental material.

α2-6 sialosides as expected (Fig. 1A), whereas wild-type (WT) VN1203 bound only α2-3 sialosides (Fig. 1B). The recombinant VN1203 virus with HA engineered to carry two RBS N224K and Q226L mutations exhibited reduced binding to avian-type receptors and increased binding to human-type receptors (Fig. 1C). Introduction of the mutation that removes the glycosylation site on the HA head (N158D) significantly increased human-type receptor binding (Fig. 1D). Addition of the stalk mutation (T318I), in either the WT or RBS mutant (N158D N224K Q226L) background, had no influence on receptor specificity (Fig. 1E and F). Direct comparison of the glycan microarray results from the whole virus with those of the corresponding recombinant HAs from VN1203 showed remarkable similarity (see Fig. S1 in the supplemental material), indicating that the specificity of the whole virus is not significantly impacted by the neuraminidase.

We then compared the HA specificities of the respiratory droplet-transmissible mutant viruses of VN1203 and INDO5. For INDO5, the engineered virus started with RBS mutations Q226L and G228S and then, during passage in ferrets, lost an N-glycosylation site at Asn158 (T160A) and acquired a stalk domain mutation (H107Y, HA1) (8). Both VN1203 and INDO5 WT HAs bind avian-type receptors only (Fig. 2A and B). With accumulation of mutations, both HAs revealed strong shifts to human-type receptor specificity. As found for VN1203, the stalk mutant in INDO5 had no significant effect on specificity (Fig. 2B and J; see also Fig. S1 in the supplemental material). While there are clear parallels in the properties conferred by the VN1203 and INDO5 mutants, there was also a significant difference in the levels of fine specificity observed in the transmissible mutants of the two strains. VN1203 RBS mutants show specificity for linear α2-6 sialosides relative to the INDO5 HA mutants, which clearly prefer α2-6 sialic acids on branched O- and N-linked glycans (glycans 47 to 53). This is evident in the engineered mutants (Fig. 2C and D) and is maintained with the additional mutations acquired during adaptation in ferrets (Fig. 2E to H).

Several reports on the crystal structures of the respiratory droplet-transmissible HA from VN1203 and INDO5 have documented the impact of the RBS mutations on receptor binding (19, 20). To gain further insights into the relationship between the RBS and stalk mutations, we compared the previously determined crystal structure of the VN1203 HA protein (Protein Data Bank [PDB] code 3GBM) with crystal structures of the HA with the three RBS mutations only and of the transmissible mutant with both RBS and stalk mutations (see Table S2 in the supplemental material). Ectodomains of the VN1203 HA were expressed in the baculovirus system essentially as previously described (21, 22). Analysis of baculovirus-produced HAs using an enzyme-linked immunosorbent assay (ELISA)-based assay system confirmed the shift in receptor specificity to human-type receptors (see Fig. S2 in the supplemental material). The overall structure of the HA trimer is shown in Fig. 3A, with the

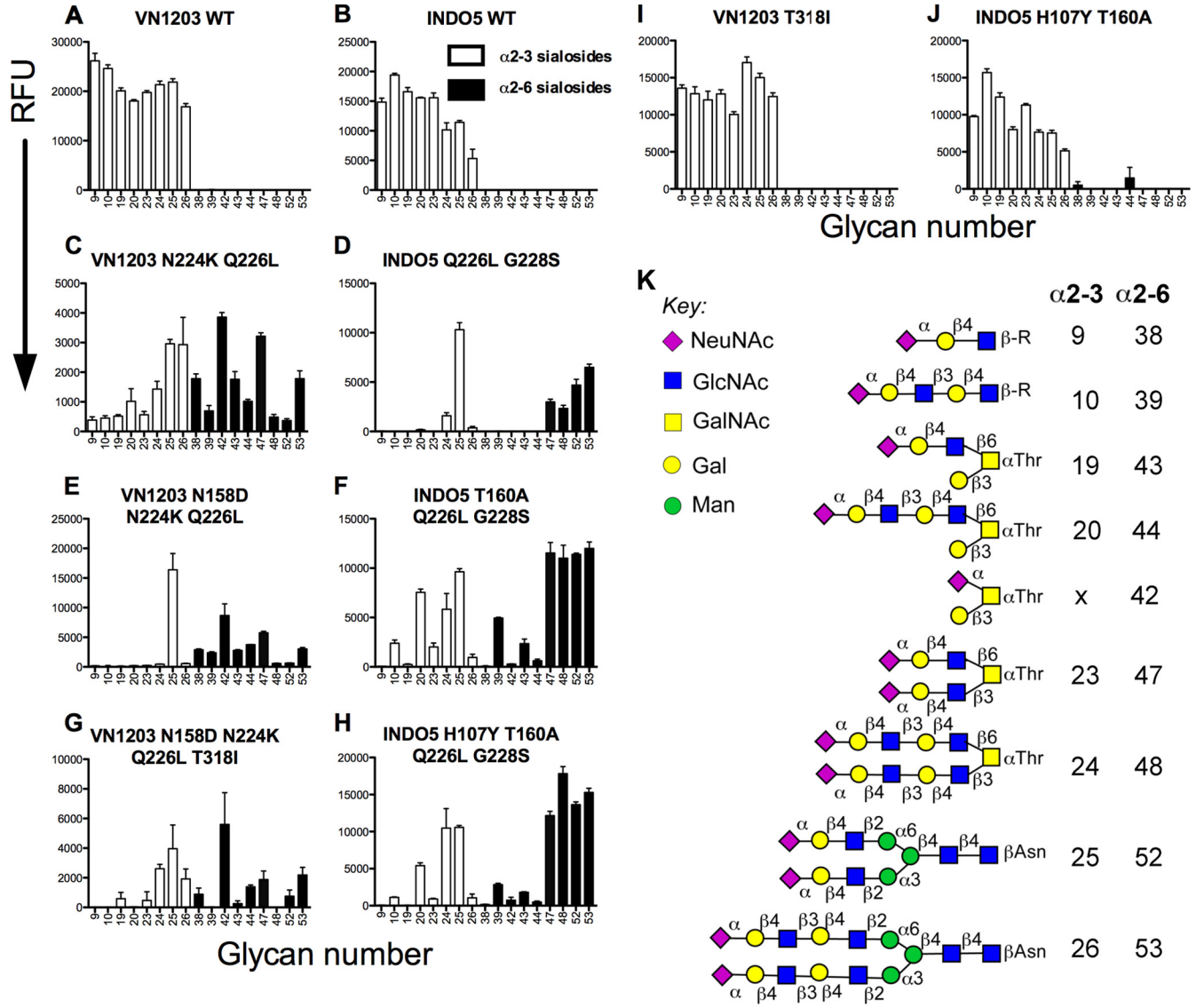

FIG 2 Specificity of respiratory droplet-transmissible recombinant VN1203 and INDO5 H5 hemagglutinins. (A to J) The receptor specificity of recombinant H5 HAs, representing a series of WT and receptor mutants of VN1203 (A, C, E, G, and I) and INDO5 (B, D, F, H, and J), was analyzed using glycan microarrays as described for Fig. 1. Results are shown for binding to selected glycans for the wild-type HAs for VN1203 (A) and INDO5 (B) and various mutants of each HA (C to J). (K) The key shows the structures of glycans selected for comparison. The data for the full array can be found in Fig. S1 in the supplemental material.

mutations indicated (see the supplemental material for methods, data collection, and refinement statistics). The RBS in the transmissible mutant is wider than its wild-type counterpart, with slight movement of loop 220 and helix 190 (~0.6 to 0.7 Å). Otherwise, the overall structures, as well as other key binding residues and elements, are virtually identical, with an overall C_α root mean square deviation (RMSD) of 0.5 Å for the HA1 domains (Fig. 3B). Despite the stability provided by the stalk mutation (5), the receptor-binding domains in the two structures with and without T318I are indistinguishable, with a C_α RMSD of 0.2 Å (Fig. 3C). Similarly, comparison of the structures with the three RBS mutations with and without T318I shows no perceptible difference in the stalk domain (Fig. 3D). The structural basis for increased stability afforded by the stalk mutations could result from an increase in hydrophobic interactions between the HA1 and HA2 domains (Fig. 3D) as the VN1203 mutant (T318I), while the H107Y stalk mutation of INDO5 creates an extra hydrogen bond between subunits of the trimer (19).

Although the stalk mutation (T318I) in VN1203 has been documented to increase the thermostability of the trimer and to shift the fusion activity of the HA to a more acidic pH (5, 19), analogous studies have not been reported for the stalk mutation (H107Y) in the INDO5 virus. To test the thermostability of this mutation in the virus, we subjected aliquots of a virus stock with the INDO5 HA in a PR8 (A/Puerto Rico/8/1934 [H1N1]) background (128 HAU) to incubation at 50°C in a heating block for various times. Aliquots were taken and then assessed in duplicate for their ability to (i) agglutinate 0.5% turkey red blood cells (TRBCs) (Fig. 4A) and (ii) grow on MDCK cells that were seeded 16 h prior to the

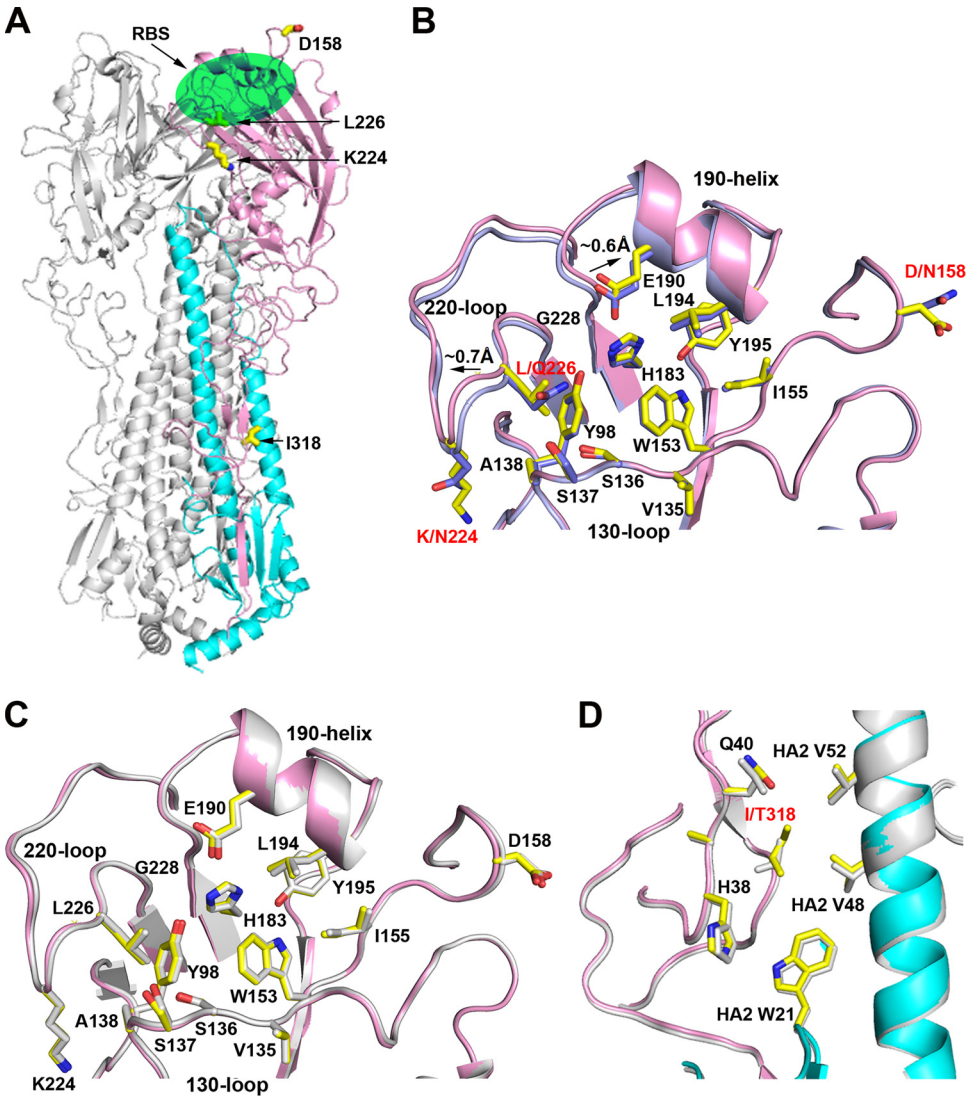

FIG 3 Crystal structures of VN1203 transmissible mutant HA (N158D, N224K, Q226L, and T318I) and its comparison with wild-type and VN1203 HA RBS mutant HA (N158D, N224K, and Q226L). (A) Overall structure of the VN1203 transmissible mutant HA. One HA protomer of the trimeric complex is colored with HA1 in pink and HA2 in cyan, with the other two protomers in gray. The four mutated HA1 residues are shown with their side chains (yellow carbon atoms, blue nitrogen atoms, and red oxygen atoms). The HA RBS is depicted in green. (B) Structural comparison of the RBSs of VN1203 transmissible mutant (with yellow side chains and pink C_α atoms) and wild-type VN1203 HA (PDB code 3GBM, with blue side chains and light blue Cα atoms). The RBS in the transmissible mutant is wider than in the wild-type HA, with slight movement of loop 220 and helix 190 by about 0.7 Å and 0.6 Å, respectively. Otherwise, the overall structures as well as key binding residues are virtually identical, with an overall Cα RMSD of 0.5 Å for the HA1 domains. (C) Structural comparison of RBSs of VN1203 transmissible mutant (with yellow side chains and pink Cα atoms) and VN1203 RBS mutant (with gray chains and Cα atoms). The RBS secondary structure elements loop 130, helix 190, and loop 220 as well as key residue side chains are virtually identical, with a Cα RMSD of 0.2 Å for HA1 domains. (D) Structural comparison of the transmissible mutant HA (with yellow side chains and pink Cα atoms) and the RBS mutant HA (with gray side chains and Cα atoms) around HA1 318. No obvious conformational changes were observed from the T318I mutation other than replacement of the side chain of 318. It appears that the T318 mutation would increase the hydrophobic interactions between HA1 and HA2 of each H5 protomer.

assay at 1.5×10^5 cells/well (Fig. 4B). Relative to the WT HA, viruses carrying the H107Y mutation showed significantly enhanced stability. Thus, while the stalk mutations of both the VN1203 and INDO5 viruses had no effect on receptor specificity, they enhance the stability of the HA trimer, which is vital for respiratory droplet transmission (5, 8).

The combined data confirm that the ferret-adapted respiratory droplet-transmissible VN1203 and INDO5 H5N1 viruses exhibit a shift to human-type specificity (5, 8, 19, 20) and further show that the two virus exhibit different fine specificities for human-type α2-6 sialosides. Using *in vitro* evolution and rational design, Chen et al. (23) reported a similar H5N1 receptor mutant (Q196R, Q226L, G228S) based on the EG06 (A/egret/Egypt/1162/2006) strain that has no N-linked glycan at N158. It exhibited an even more extreme shift to specificity for α2-6 sialosides than those reported here and yet supported only contact transmission, but not respiratory droplet transmission, in ferrets. In this case, partial respiratory droplet transmission was achieved by substituting a human N2 neuraminidase in the

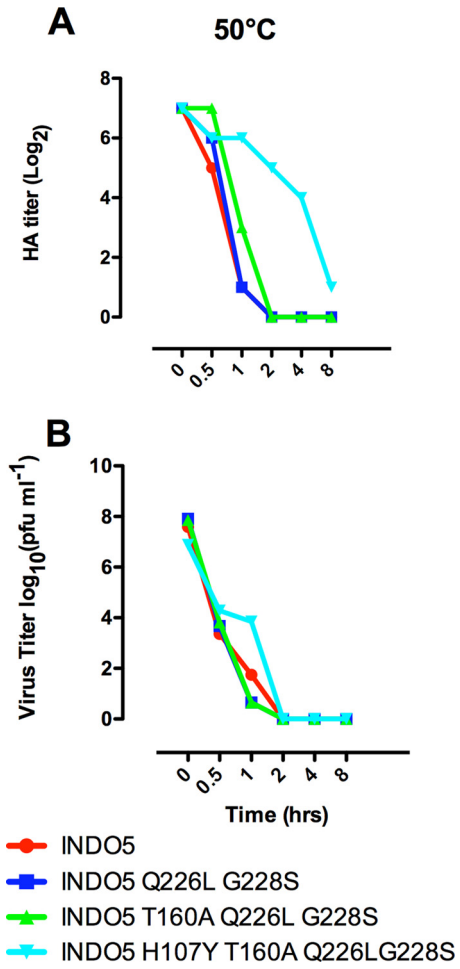

FIG 4 Stability conferred by the H107Y mutation in INDO5 HA-containing viruses. The stability of INDO5 and selected mutants was assessed after incubation of the virus over time at 50°C. (A) Hemagglutination of turkey erythrocytes with treated virus. (B) Viral growth of treated virus in MDCK cells. Results are representative of two independent analyses.

EG06 (23). Thus, while acquisition of human-type receptor specificity clearly plays an important role in transmission in the ferret model, a balance of other factors, including receptor avidity, trimer stability and fusion activity, and neuraminidase activity, is needed to achieve a transmissible virus (7).

Protein structure accession numbers. The atomic coordinates and structure factors determined in this work have been deposited in the Protein Data Bank (PDB) under accession codes 4N5Y for the VN1203 RBS mutant HA and 4N5Z for the VN1203 transmissible mutant HA.

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